A hierarchy of ATP-consuming processes in mammalian cells

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The rates of different ATP-consuming reactions were measured in concanavalin A-stimulated thymocytes, a model system in which more than 80 % of the ATP consumption can be accounted for. There was a clear hierarchy of the responses of different energy-consuming reactions to changes in energy supply: pathways of macromolecule biosynthesis (protein synthesis and RNA/DNA synthesis) were most sensitive to energy supply, followed by sodium cycling and then calcium cycling across the plasma membrane. Mitochondrial proton leak was the least sensitive to energy supply. Control analysis was used to quantify the relative control over ATP production exerted by the individual groups of ATP-consuming reactions. Control was widely shared; no block of reactions had more than one-third of the control. A fuller control analysis showed that there appeared to be a hierarchy of control over the flux through ATP: protein synthesis > RNA/DNA synthesis and substrate oxidation > Na^+ cycling and Ca^{2+} cycling > other ATP consumers and mitochondrial proton leak. Control analysis also indicated that there was significant control over the rates of individual ATP consumers by energy supply. Each ATP consumer had strong control over its own rate but very little control over the rates of the other ATP consumers.

INTRODUCTION

Meyerhof in 1945 [1] was perhaps the first to demonstrate that consumption of ATP is necessary for steady-state ATP production without accumulation of glycolytic intermediates. Since then it has widely been assumed that cellular respiration and ATP synthesis are controlled by cellular ATP utilization, i.e. that mitochondria passively respond to changes in ATP demand. However, there is now a growing body of evidence that the rates of some ATP-utilizing reactions may be controlled by mitochondrial activities, i.e. mitochondrial ATP supply can contribute actively to control of ATP consumption [2–4].

Atkinson [5] felt that 'It seems likely that there is a hierarchy of such (ATP-consuming) processes in terms of their responses to the value of the energy charge. Energy-storing sequences, such as the syntheses of polysaccharide or fat, should be most sensitive to decrease in energy charge. Biosyntheses of structural macromolecules should be next, and activities that are essential for maintenance of life should be able to function at lower values of charge'. There is scattered evidence that such a hierarchy does exist: different authors have described the sensitivity of individual processes to availability of metabolic energy. For example, Mendelsohn et al. [6] and Gronostajski et al. [7] reported protein synthesis to be very sensitive to energy supply, and there are indications that ion transport is less sensitive to the [ATP]/[ADP] ratio (see [4]).

In the present paper we examine ATP consumption in concanavalin A (Con A)-stimulated rat thymocytes. These cells represent a model system in which we have identified the reaction pathways responsible for more than 80 % of the ATP consumption [8,9]. We present experimental evidence that individual ATP-consuming processes respond very differently to energy supply, producing a hierarchic ordering of the energy-consuming pathways. This hierarchy has not previously been demonstrated explicitly for a range of major ATP-consuming pathways in a single system. We then use control analysis to show how strongly each major ATP-consuming reaction controls the flux through ATP and to indicate how strongly each major ATP-consuming reaction is controlled by energy supply and by the other ATP-consuming reactions.

MATERIALS AND METHODS

Preparation and incubation of cells

Thymocytes were prepared daily from a 4–6-week-old female Wistar rat as described previously [9]. The thymus was disaggregated by pressing it through nylon mesh. The cells were centrifuged once at 1000 *g* for 5 min and resuspended at (5–6) × 10^7 cells/ml at 37 °C. Isolation and incubation medium was RPMI 1640 (obtained powdered from Flow Laboratories) containing 10 mM glucose (unless stated otherwise) and 2 mM glutamine, buffered at pH 7.4 with 10 mM Hepes and 24 mM $NaHCO_3$; this was supplemented with 20 μg of gentamicin/ml and filtered through a 0.2 μm-pore-size filter to remove undissolved particles. Cells were stored and incubated for up to 4 h in plastic flasks at 37 °C with gentle shaking (80 cycles/min) under an atmosphere of CO_2/air (1:19). The viability of freshly isolated cells was greater than 95 %, as determined by Trypan Blue exclusion.

Measurements of respiration rate

Oxygen consumption was measured amperometrically in a 1.0 ml aliquot of cell suspension with a Clark electrode for up to 15 min as described previously [9]. The cell suspensions in the Perspex

Abbreviations used: Con A, concanavalin A; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; J_{ATP}, flux through ATP in units of the oxygen consumption used to drive it; C, control coefficient, defined as the fractional change in a variable (usually flux) caused by an infinitesimal fractional change in a system parameter when the system is allowed to relax to a new steady state; ϵ, elasticity, defined as the fractional change in a local flux caused by an infinitesimal fractional change in a system variable when the system is not allowed to relax; $\Delta\psi$, mitochondrial membrane potential.

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incubation chamber of the electrode were magnetically stirred and thermostatically maintained at 37 °C. Inhibitors were added in water or in DMSO. Con A (ICN ImmunoBiologicals, Lisle, Israel) was dissolved in water and added to cell suspensions at 25 μg/ml. We applied inhibitors of protein synthesis (cycloheximide), Na^+/K^+-ATPase (ouabain), Ca^{2+}-ATPase ($LaCl_3$) and RNA/DNA synthesis (actinomycin) at the following final concentrations as described previously [8–10]: cycloheximide, 1 mM; ouabain, 1 mM; $LaCl_3,7H_2O$, 2 mM; actinomycin D, 0.6 mM; oligomycin, 5–60 ng/ml; rotenone, 20–80 nM (all from Sigma Chemical Co., Dorset, U.K.); myxothiazol (Boehringer, Mannheim, Germany), 7.5–17.5 nM; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Aldrich Chemical Co., Dorset, U.K.), 10–100 nM. Respiration rates were measured 1–3 min after addition of Con A or inhibitor.

RESULTS AND DISCUSSION

A hierarchy of energy consumers in thymocytes

The basal respiration rate of quiescent thymocytes was 4.12 ± 0.19 nmol of O_2/min per 5×10^7 cells. Addition of Con A stimulated this within seconds by about 31 % to 5.41 ± 0.18 nmol of O_2/min per 5×10^7 cells (mean ± S.E.M. for 16 cell preparations). We identified and quantified the processes consuming ATP in these Con A-stimulated cells by measuring the decrease in cellular oxygen consumption caused by inhibition of particular ATP-consuming processes, and we calculated the oxygen consumption used to drive a futile cycle of proton pumping and proton leak across the mitochondrial inner membrane from data obtained previously under identical conditions [9]. Table 1 shows the rates of different ATP-consuming reactions and of the proton leak in Con A-stimulated thymocytes. Together these identified processes account for 94 % of the respiration rate of the cells. Since non-mitochondrial oxygen consumption is negligible in thymocytes [11], the remaining 6 % can be assigned to unidentified ATP-consuming processes. These data are similar to those we have described previously in this and other media, where we accounted for more than 80 % of cellular respiration, with proton leak contributing 20–40 % of the total [8–10].

We then investigated the effect of restricting mitochondrial ATP production by progressively inhibiting the electron transport chain using myxothiazol, which inhibits complex III. Figure 1 shows the inhibitory effect of myxothiazol on respiration of Con A-stimulated thymocytes. At each myxothiazol concentration we remeasured the contribution of different energy-consuming processes to respiration rate as above. Figure 2 shows the results, expressed arbitrarily as a semi-logarithmic plot, so that we could apply linear regressions and statistical tests (see below). Protein and polynucleotide syntheses were most sensitive to inhibition of respiration, their rates falling by 60 % when respiration was inhibited by 30 %. Other processes were less sensitive to energy supply: at the same respiration rate sodium cycling through the Na^+/K^+-ATPase was inhibited 45 %, calcium cycling through the plasma membrane Ca^{2+}-ATPase was inhibited 30 % and proton cycling across the mitochondrial inner membrane was inhibited 20 %. We tested for differences between the five regression lines in Figure 2 by analysis of co-variance [12]. All regression lines except those for protein synthesis and

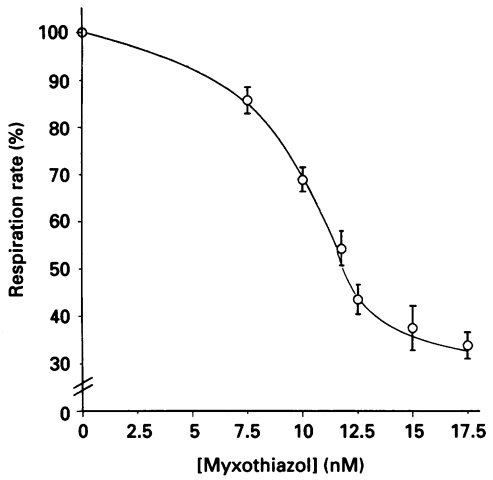

Figure 1 Effects of myxothiazol on respiration of Con A-stimulated thymocytes

Thymocytes were incubated in RPMI medium with 25 μg Con A/ml, followed by application of different concentrations of myxothiazol. Respiration rate was assayed as described in the Materials and methods section. Results are means ± S.E.M. for 11 cell preparations.

Table 1 Rates of different energy-consuming reactions in Con A-stimulated thymocytes and their control over system variables

Rates of specific ATP consumers were measured as the decrease in respiration rate caused by complete inhibition of the process, assuming that such inhibition did not alter the rates of other processes (justified in the text). J_{ATP} is the rate of ATP synthesis and consumption in units of oxygen consumption rate; equal to 3.3 nmol of O_2/min per 5×10^7 cells. $C^{J_{ATP}}$ (or J_S, J_L, P/O, $\Delta\psi$) are the flux control coefficients of different blocks of reactions over J_{ATP} (or substrate oxidation rate, proton leak rate, effective P/O ratio or $\Delta\psi$), calculated as described in the text. The fraction of J_{ATP} that is used by each process is equal to the fraction of the total control by the ATP consumers over the rate of ATP production exerted by that process.

Process driven by respiration	Process rate: nmol of O_2/min per 5×10^7 cells ± S.E.M.	Process rate: % of total respiration rate	Fraction of J_{ATP}	$C^{J_{ATP}}$	C^{J_S}	C^{J_L}	$C^{P/O}$	$C^{\Delta\psi}$
Protein synthesis	1.11 ± 0.12 ($n = 9$)	20.5	0.34	0.30	0.17	−0.03	0.13	−0.02
Na^+/K^+-ATPase	0.52 ± 0.07 ($n = 9$)	9.6	0.16	0.14	0.08	−0.01	0.06	−0.01
Ca^{2+}-ATPase	0.55 ± 0.12 ($n = 6$)	10.2	0.17	0.15	0.08	−0.01	0.06	−0.01
RNA/DNA synthesis	0.81 ± 0.18 ($n = 5$)	15.0	0.25	0.21	0.12	−0.02	0.09	−0.01
Unidentified ATP consumers*	0.31	5.7	0.09	0.08	0.05	−0.01	0.04	−0.00
Proton leak†	2.11 ± 0.20	39.0						
Sum	5.41 ± 0.18 ($n = 16$)	100	1.01	0.88	0.50	−0.08	0.38	−0.05

* Calculated as the difference between total respiration rate and the sum of the identified reactions.

† Scaled from data obtained under identical conditions in [9].

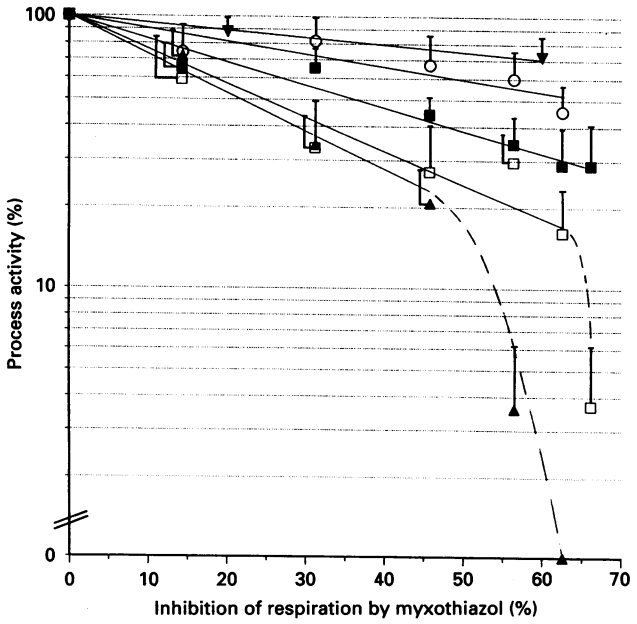

Figure 2 Effects of myxothiazol on ATP-consuming processes and proton leak

Thymocytes were incubated in RPMI medium with 25 μg Con A/ml and different concentrations of myxothiazol, followed by various inhibitors of ATP-consuming processes at the concentrations reported in the Materials and methods section. Process activity is given as a percentage of the activity in the absence of myxothiazol. Results are means ± S.E.M. for 5–9 cell preparations. Regression lines are drawn through all points above 10% activity. There was a good fit in all cases (F test). ▲, Process inhibited by cycloheximide (protein synthesis); □, process inhibited by actinomycin (RNA/DNA synthesis); ■, process inhibited by ouabain (Na^+ cycling); ○, process inhibited by La^{3+} (Ca^{2+} cycling); ▼, proton leak (data for proton leak from [9]).

RNA/DNA synthesis differed significantly from each other ($P < 0.05$).

Thus there is a clear hierarchy of energy-consuming processes in these cells: pathways of macromolecule biosynthesis (protein synthesis and RNA/DNA synthesis) are the most sensitive to ATP supply, followed by sodium cycling and then by calcium cycling across the plasma membrane. Mitochondrial proton leak is the process least sensitive to energy supply. The hierarchy of ATP-consumers is consistent with that predicted by Atkinson [5].

Two controls were carried out to determine whether these results reflect a general pattern in regulation of energy metabolism. To ensure that glycolytic ATP production was not affecting the results, we repeated the experiments in the absence of glucose, conditions in which the production of lactate is negligible in thymocytes [8]. We found the same hierarchy as for the complete medium (results not shown). Secondly, we replaced myxothiazol with either another respiratory chain inhibitor (rotenone), an inhibitor of the mitochondrial ATP-synthase (oligomycin) or an uncoupler (FCCP). In each case protein synthesis was more sensitive to reduced energy supply than was Na^+/K^+-ATPase (results not shown), confirming the results obtained using myxothiazol.

In these and previous experiments [8–10] we calculated the rates of individual ATP-consuming reactions from the effects of complete inhibition of these processes on respiration. The calculations rely on the assumption that inhibition of one ATP-consuming process does not significantly stimulate the rates of the others through increases in cellular ATP levels. If this assumption is wrong it would lead to an underestimate of the rate of the process. Our conclusion that there is a hierarchy of responses of ATP-consuming reactions to energy supply implies that at least some of the pathways must be significantly sensitive to ATP, and the assumption must to some extent be untrue. In addition, not only will other reactions increase, but those that are most sensitive to ATP will increase more than the others. However, the calculated flux control coefficients of the identified ATP consumers over each other and over the mitochondrial proton leak are very small (−0.05 or less; see below) and the individual ATP-consuming processes account for only 20% or less of the respiration rate (Table 1), so the systematic errors generated by this assumption are also likely to be small. In principle we could make a correction for such errors by reiteratively recalculating the rates from the elasticities and control coefficients, but in practice the experimental errors in the present data would make such a calculation untrustworthy.

Control of energy metabolism in thymocytes

The data that we have collected are sufficient to allow a control analysis (see [4,13–23]) of ATP consumption in Con A-stimulated thymocytes. Control analysis of this system can give a quantitative description of the distribution of control over the flux through ATP in an intact cell, to complement and significantly extend previous descriptions of control over the production and consumption of mitochondrial potential in thymocytes [9], hepatocytes [2,3] and intact muscle [20] and over NADH production and consumption in hepatocytes [2]. All control coefficients (C) presented in the present paper refer to the uninhibited state; the values in the presence of the inhibitors will be different.

Control over the reactions around cytoplasmic ATP: ATP producers and ATP consumers

First, we calculated the control over ATP production rate exerted by each of the individual blocks of ATP-consuming reactions (protein synthesis, sodium cycling, calcium cycling, RNA/DNA synthesis and unidentified ATP consumers). By subtracting the oxygen consumption used to drive the proton leak [9] from the total oxygen consumption rate, we calculated the rate of ATP production in terms of the oxygen consumption needed to drive it (glycolytic ATP production is negligible under our conditions). From the measured rates of the ATP consumers we then calculated the fraction of ATP production used by each of the individual blocks of ATP-consuming reactions (Table 1). From the branching theorem of control analysis [21] we know that control over the rate of ATP production is divided among the ATP consumers in the ratio of their fluxes. The reactions with the largest fluxes had the greatest share of the control over ATP production rate (Table 1).

Control over the reactions around mitochondrial membrane potential: substrate oxidation, proton leak and the phosphorylating system

The conclusion reached in the previous section is useful, but we can go further. From our previous work under identical experimental conditions [9] we know that the phosphorylating system that consumes mitochondrial membrane potential ($\Delta\psi$) has a control coefficient of 0.88 over the flux through ATP (J_{ATP}),

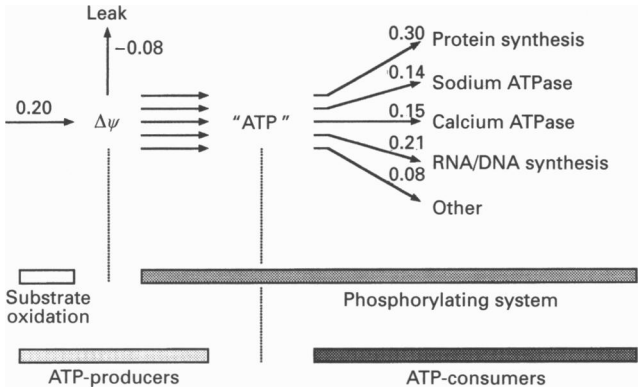

Figure 3 Control over flux through cytoplasmic ATP (J_{ATP})

The reactions of ATP production and consumption are divided around the mitochondrial membrane potential, $\Delta\psi$, into substrate oxidation (all reactions between the oxidizable substrate and the mitochondrial redox proton pumps) and the phosphorylating system (all reactions of ATP synthesis and utilization) [9], or around cytoplasmic ATP (or some undefined unique function of it, such as the ATP/ADP ratio or the phosphorylation potential, see [15]) into ATP producers and ATP consumers, as indicated by the bars. The control coefficients of the different reactions over the flux through ATP, J_{ATP}, from Table 1, are indicated above each reaction. The analysis assumes that none of the metabolites within any block of reactions affects any other block except through the explicit intermediates [15].

leaving 0.12 for the control by the rest of the system (divided 0.20 to substrate oxidation and −0.08 to the mitochondrial proton leak). The control over J_{ATP} by the phosphorylating system is now divided among the individual ATP consumers according to their fluxes as shown in Table 1.

In the same way, the individual ATP-consuming reactions share the control over substrate oxidation rate (J_S), proton leak rate (J_L), effective P/O ratio and mitochondrial potential, the other variables whose control was analysed in [9]. Control over these variables by the phosphorylating system as a whole is also divided in the ratio of the fluxes through the individual ATP-consuming reactions as shown in Table 1.

Thus control over J_{ATP} in these Con A-stimulated thymocytes is distributed as summarized in Figure 3. Control is widely shared; no block of reactions has more than one-third of the control. The hierarchy of control over flux through ATP is protein synthesis (0.3) > RNA/DNA synthesis and substrate oxidation (0.2) > Na^+ cycling and Ca^{2+} cycling (0.15) > unidentified ATP consumers and mitochondrial proton leak (0.1 and −0.1).

Control over flux through individual ATP consumers

Finally, we calculated the control over the flux through each of the individual ATP-consuming branches exerted by the other blocks of reactions that make up the system around $\Delta\psi$. Briefly, to do this we estimated the relative elasticities of the individual ATP consumers to $\Delta\psi$ from the data used to generate Figure 2, converted the relative elasticities to absolute elasticities using eqn. (1) and the overall elasticity of the phosphorylating system in [9] and the control coefficients over J_{ATP} in Table 1, then solved the usual type of equations [16,22] [eqns. (2) and (3)] to calculate the full set of control coefficients from the elasticities and fluxes (Table 2). The calculations are described more fully in the following paragraphs.

Values for the absolute elasticities to $\Delta\psi$ of substrate oxidation (−11.0), proton leak (1.8) and the phosphorylating system (2.6) were taken from [9]. The value of −19.1 for the elasticity of the grouped $\Delta\psi$-producers (substrate oxidation plus proton leak) was calculated from the relationship between the overall elasticity of a block to $\Delta\psi$ and the elasticities of its i constituent reactions, derived in [23]:

$$\epsilon^{block}_{\Delta\psi} = \sum_{i=1}^{n} C^{block}_{i}\,\epsilon^{i}_{\Delta\psi} \qquad (1)$$

The relative elasticities of the individual ATP consumers to $\Delta\psi$ and to cytoplasmic ATP (and to other unique but undefined functions of ATP such as ATP/ADP ratio or phosphorylation potential) in Table 2 were estimated from the relative changes in their rates when $\Delta\psi$ and cytoplasmic ATP concentration were altered by inhibiting $\Delta\psi$ and ATP production by adding myxothiazol (Figure 2). Since addition of a particular amount of myxothiazol causes specific (but unmeasured) changes in $\Delta\psi$ and in ATP, the fractional changes in the rates of the individual ATP consumers are proportional to their elasticities to both $\Delta\psi$ and ATP. The relative elasticities in Table 2 were calculated as the mean measured values of $\Delta J_{process}/J_{process}$ at 7.5, 10 and 12.5 nM myxothiazol, using the raw data points to avoid assumptions about the shape of the inhibition curves. Slightly different values would have been obtained if we had assumed an exponential relationship and simply taken the relative elasticities from the

Table 2 Control by different ATP-consuming reactions over each other

Relative elasticities were calculated as the mean fractional inhibition of process activity caused by addition of 7.5, 10 and 12.5 nM myxothiazol as shown in Figure 2, scaled to the elasticity of protein synthesis to $\Delta\psi$ and ATP. The small rate of the unidentified ATP consumers at each myxothiazol concentration is calculated by subtracting the sum of the identified rates from the total rate, hence its value is very error-prone. The calculated relative elasticity of −1.80 for the unidentified processes to $\Delta\psi$ was not significantly different from zero and was therefore ignored; a value of 0.00 was arbitrarily assumed. This assumption made little difference to the values of the control coefficients below. Absolute elasticities to $\Delta\psi$ were calculated from relative elasticities as described in the text. Control coefficients of the different processes over the individual ATP consumers were calculated from elasticities and fluxes using eqns. (2) and (3) as described in the text.

Process	Relative elasticity to $\Delta\psi$ and ATP	Absolute elasticity to $\Delta\psi$	$C^{Protein\ synth}$	$C^{Na\ cycle}$	$C^{Ca\ cycle}$	$C^{RNA/DNA\ synth}$	C^{Other}
$\Delta\psi$ production		−19.1	0.15	0.12	0.08	0.16	0.00
$\Delta\psi$ consumption		2.6					
Protein synthesis	1.00	3.15	0.95	−0.04	−0.03	−0.05	0.00
Na^+/K^+-ATPase	0.83	2.59	−0.02	0.98	−0.01	−0.03	0.00
Ca^{2+}-ATPase	0.52	1.65	−0.02	−0.02	0.99	−0.03	0.00
RNA/DNA synthesis	1.10	3.48	−0.04	−0.03	−0.02	0.96	0.00
Unidentified ATP consumers	0.00	0.00	−0.01	−0.01	−0.01	−0.02	1.00

slopes in Figure 2, but such an assumption is not supportable. Ideally, the relative elasticities should have been obtained from the tangents of the inhibition curves at zero myxothiazol, but the errors in the data made that impossible.

The absolute elasticities of the individual ATP consumers to $\Delta\psi$ were then calculated from the relative elasticities using eqn. (1), incorporating the values for the flux control coefficients of each ATP consumer over the phosphorylating system flux (equal to the fraction of J_{ATP} passing through that consumer) from Table 1 and the absolute value of 2.6 for the overall elasticity of the phosphorylating system to $\Delta\psi$.

It is straightforward to calculate control coefficients from elasticities and fluxes [16,22]. Using the summation, connectivity and branching theorems we derived the following equations and used them to calculate the control coefficients shown in Table 2. To keep the equations general, we changed sign convention for the calculations: fluxes towards the intermediate were defined as positive, so that the ATP consumers had negative fluxes and therefore negative elasticities to $\Delta\psi$. However, all values of elasticities and fluxes quoted in the present paper use the original sign convention, with fluxes from left to right in Figure 3 defined as positive. For n branches at a common intermediate M, where fluxes towards the intermediate are defined as positive, the control of a branch over its own flux is given by:

$$C_a^{J_a} = \left(\sum_{i=1}^{n} J_i \epsilon_M^i - J_a \epsilon_M^a\right) \Big/ \sum_{i=1}^{n} J_i \epsilon_M^i \qquad (2)$$

and control of a branch over the flux through a different branch is given by:

$$C_a^{J_b} = J_a \epsilon_M^b \Big/ \sum_{i=1}^{n} J_i \epsilon_M^i \qquad (3)$$

Eqn. (2) is the same as eqns. (7) and (10) derived in [24] but generalized to n branches and using the changed sign convention {see also eqn. (15) in [15] and eqn. (1) in [16]}. Eqn. (3) is the same as eqn. (6) derived in [24] but generalized to n branches and using the changed sign convention {see also eqns. (4) and (7) in [16]}.

The results in Table 2 show that there is control over the rates of individual ATP consumers by $\Delta\psi$ production (energy supply). Protein synthesis and RNA/DNA synthesis are significantly controlled by energy supply (C = approx. 0.2) whereas ion cycling is less strongly controlled (C = approx. 0.1). The control of energy supply over macromolecule synthesis and lower control over ion cycling agrees with and quantifies the hierarchy of ATP consumers identified above.

The control coefficients of individual ATP consumers over their own rates are all above 0.95, showing that they are very strongly controlled by their own activities. The control over any individual ATP consumer by the other identified ATP consumers is extremely small, −0.05 or less, supporting our original assumption that inhibition of one process will not greatly affect the rates of the others.

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Conclusions

These results clearly show that in Con A-stimulated thymocytes there is a hierarchy of ATP-consuming reactions, with macromolecule synthesis quite strongly controlled by ATP supply and ion cycling less strongly controlled. As discussed by Atkinson [5] and Brown [4], this hierarchy means that the fate of ATP generated by a cell can be determined by the ATP-producing reactions as well as by the ATP consumers. In addition, processes that are not essential for the immediate needs of the cell will be given up before those that are more critical for ionic integrity if ATP supply is compromised.

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